

Prevalence of species and genotypes of *Cryptosporidium* found in 1–2-year-old dairy cattle in the eastern United States

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Abstract

The prevalence of *Cryptosporidium* species in 1–2-year-old heifers was determined for 571 animals on 14 dairy farms in seven states on the East Coast of the United States. A fecal specimen collected directly from each heifer was processed to concentrate oocysts that were then examined by polymerase chain reaction (PCR). For every PCR-positive specimen the 18S rRNA gene of *Cryptosporidium* was sequenced. *Cryptosporidium* was identified by PCR from heifers on 13 of 14 farms. On all except four farms groups of heifers were housed in a barn or in large covered pens. Others were pastured. From many of the same farms an earlier study reported that 41% of 393 pre-weaned calves and 26.2% of 447 post-weaned calves were infected. In the present study, 11.9% of 571 heifers were infected with *Cryptosporidium*, 0.7% with *Cryptosporidium parvum*, the zoonotic species. Of 68 PCR-positive specimens characterized by gene sequencing 1, 4, 10, 24, and 29 calves were infected with *Cryptosporidium suis*, *Cryptosporidium parvum*, *Cryptosporidium* deer-like genotype, *Cryptosporidium bovis*, and *Cryptosporidium andersoni*, respectively. These findings demonstrate a lower prevalence of infection in 1–2-year-old dairy cattle than in younger cattle as well as a change in the diversity of species present. Consequently, the risk of humans acquiring infection with *C. parvum* from exposure to feces from yearling and older cattle appears much lower than from exposure to pre-weaned calves. Published by Elsevier B.V.

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1. Introduction

Three species of *Cryptosporidium* with marked biological differences infect cattle: *Cryptosporidium*

parvum, *Cryptosporidium andersoni* (formerly known as *Cryptosporidium muris*; Lindsay et al., 2000), and *Cryptosporidium bovis* (formerly known as *Cryptosporidium* genotype bovine B; Fayer et al., 2005). *Cryptosporidium parvum*, infect the small intestine primarily of pre-weaned calves as well as humans and other animals, often causing diarrheal disease (Case-more et al., 1997; Fayer et al., 1997, 2000a; Morgan

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et al., 1999; Santín et al., 2004). *Cryptosporidium andersoni*, infects the abomasum of juvenile and mature cattle; infection has been implicated as a cause of reduced milk production but has not been associated with other signs of disease (Olson et al., 1997; Anderson, 1998; Lindsay et al., 2000). Recently, *C. bovis* was found to be the predominate species infecting 2–11-month-old dairy calves but was not associated with overt disease (Santín et al., 2004). A fourth *Cryptosporidium*, referred to as the deer-like genotype, has been reported from both pre-weaned and 2–11-month-old calves also without associated signs of disease (Santín et al., 2004).

Although identification of *Cryptosporidium* in cattle based on oocyst morphology (Quílez et al., 1996; Uga et al., 2000; Wade et al., 2000; Zorana et al., 2001; Castro-Hermida et al., 2002a,b; Kvac and Vitovec, 2003) and immunofluorescence microscopy (Xiao et al., 1993; Xiao and Herd, 1994; Olson et al., 1997; Atwill et al., 1999; O'Handley et al., 1999; Fayer et al., 2000b; Sisco et al., 2000; Ralston et al., 2003; Sturdee et al., 2003) provides evidence for the presence of *Cryptosporidium*, neither method is capable of differentiating the species or genotypes of *Cryptosporidium* and therefore cannot associate disease or risk of human infection with the presence of oocysts (Fayer et al., 2000a; Egyed et al., 2003; Monis and Thompson, 2003). Utilizing molecular techniques, the prevalence of each of the aforementioned species and genotypes of *Cryptosporidium* was determined for 840 dairy calves from birth to 11 months of age and differences were found in the prevalence of each species and genotype relative to the age of the animals (Santín et al., 2004). For pre-weaned and post-weaned calves *C. parvum* was identified in 85% and 1% of the positive specimens, respectively. In contrast, *C. bovis* (Fayer et al., 2005), previously identified as *Cryptosporidium* genotype bovine B, was found in 9% and 55% of the positive specimens from pre-weaned calves and in post-weaned calves, respectively. Likewise, *C. andersoni*, was found in 1% and 13% of similar specimens. The aforementioned findings come from molecularly based, geographically widespread studies involving large numbers of cattle, and provide data that are potentially important for public health, manure management, and herd management decisions. Similar data are not available for older dairy cattle. Therefore, the present study was undertaken to precisely identify the species and genotypes of *Cryptosporidium* in 1–2-year-old

dairy cattle on farms over a multi-state area encompassing much of the East Coast of the United States.

2. Material and methods

2.1. Sources and collection of specimens

Feces were collected from 571 female cattle (heifers) 12–24 months of age, on two dairy farms in each of the following states: Pennsylvania, Vermont, New York, Maryland, Virginia, North Carolina, and Florida (Table 1). All farms were visited once in 2004 and the number of heifers on each farm that provided fecal specimens is shown in Table 1. Ten farms in a previous study (Santín et al., 2004) were revisited but management changes required replacement of farms previously designated as PA-2, VA-1, NC-1, and NC-2 with farms PA-3, VA-3, NC-3, and NC-4, respectively.

All specimens were collected from heifers housed in groups. Heifers were confined to large pens with cement floors partially or fully covered by a roof except on farms FL-1, FL-2, VA-3, and MD-2 where heifers grazed in open fields, and farm VA-2 where heifers were confined in a large free-stall barn with access to an adjacent pen.

Feces were collected directly from the rectum of each heifer into a plastic cup. Cups were capped, labeled with the heifer's ear tag number, and immediately placed in an insulated container packed with ice or cold packs. Specimens were transported to the USDA laboratory in Beltsville, MD and processed within 1–3 days of collection as previously described (Santín et al., 2004).

2.2. Oocyst concentration from feces

Oocysts were concentrated from feces as previously described (Fayer et al., 2000b; Santín et al., 2004). Briefly, 15 g of feces from each specimen cup were mixed with 35 ml dH₂O. The suspension was passed through a sieve with a 45 µm pore size screen. The filtrate volume was adjusted to 50 ml with dH₂O and centrifuged at 1800 × g for 15 min. The pellet was resuspended in a mixture of 25 ml dH₂O and 25 ml CsCl (1.4 g/l) and centrifuged at 300 × g for 20 min. Supernatant, aspirated from each suspension, was washed

Table 1

Farm designation, number of heifers examined at each farm, and number of positives and prevalence of *Cryptosporidium* species/genotypes in by PCR from 12–24-month-old dairy heifers on 14 farms in seven states

State	Farm	n	Number of positives (Prevalence)				
			<i>Cryptosporidium</i>	<i>C. parvum</i>	<i>C. bovis</i>	Deer-like	<i>C. andersoni</i>
VT	VT-2	31	3 (9.7)	0 (0)	0 (0)	3 (9.7)	0 (0)
	VT-3	39	3 (7.7)	1 (3)	2 (5.1)	0 (0)	0 (0)
NY	NY-1	34	3 (8.8)	0 (0)	0 (0)	1 (2.9)	2 (5.9)
	NY-2	37	6 (16.2)	0 (0)	1 (2.7)	0 (0)	5 (13.5)
PA	PA-1	62	7 (11.3)	0 (0)	1 (1.6)	2 (3.2)	3 (4.8)
	PA-3	53	8 (15.1)	1 (1.9)	5 (9.4)	0 (0)	2 (3.8)
MD	MD-1	50	2 (4)	0 (0)	1 (2)	0 (0)	1 (2)
	MD-2	43	7 (16.3)	0 (0)	1 (2.3)	0 (0)	6 (14)
VA	VA-2	42	12 (28.6)	0 (0)	10 (23.8)	2 (5)	0 (0)
	VA-3	29	1 (3.4)	0 (0)	0 (0)	0 (0)	1 (3.4)
NC	NC-3	36	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	NC-4	29	7 (24.1)	0 (0)	0 (0)	0 (0)	7 (24.1)
FL	FL-1	47	7 (14.9)	2 (4.3)	3 (6.4)	2 (4.3)	0 (0)
	FL-2	39	2 (5.1)	0 (0)	0 (0)	0 (0)	2 (5)
Total		571	68 (11.9)	4 (0.7)	24 (4.2)	10 (1.8)	29 (5.1)

The values in parenthesis are in percentage. One calf infected with *C. suis* in PA-1.

twice with dH₂O and the final pellet was suspended in 500 µl of dH₂O. The suspension was examined by molecular methods as described below.

2.3. DNA extraction

Total DNA was extracted from each CsCl-cleaned fecal sample using a DNeasyTissue Kit (Qiagen, Valencia, CA) with a slightly modified protocol. The protocol, described below, utilized reagents provided by the manufacturer. A total of 50 µl of processed feces were suspended in 180 µl of ATL buffer and thoroughly mixed by vortexing. To this suspension, 20 µl of proteinase K (20 mg/ml) was added, and the sample was thoroughly mixed. Following an overnight incubation of the mixture at 55 °C, 200 µl of AL buffer was added. The remaining protocol followed manufacturer's instructions with one exception. To increase the quantity of recovered DNA, the nucleic acid was eluted in 100 µl of AE buffer.

2.4. Gene amplification and sequencing

A two-step nested PCR protocol was used to amplify an 830 bp fragment of the 18S rRNA gene using primers

5'-TTCTAGAGCTAATACATGCG-3' and 5'-CCCAT-TTCCTTCGAAACAGGA-3' for primary PCR and 5'-GGAAGGGTTGTATTT-ATTAGATAAAG-3' and 5'-AAGGAGTAAGGAACAACCTCCA-3' for secondary PCR (Xiao et al., 1999). The primary PCR mixture contained 1× PCR buffer, 3 mM MgCl₂, 0.2 mM dNTP, 2.5 U Taq (Qbiogene, Irvine, CA), 2.5 µl BSA (0.1 g/10 ml), and 1 µM of each forward and reverse primer in a 50 µl reaction volume. Each of 35 cycles consisted of 94 °C for 45 s, 59 °C for 45 s, and 72 °C for 1 min after an initial hot start at 94 °C for 3 min and ending with 72 °C for 7 min. The secondary PCR mixture was identical except that the MgCl₂ concentration was 1.5 mM. Each of 40 cycles consisted of 94 °C for 30 s, 58 °C for 90 s, and 72 °C for 2 min after an initial hot start at 94 °C for 3 min and ending with 72 °C for 7 min. PCR products were analyzed on 1% agarose gel and visualized after ethidium bromide staining.

2.5. Sequence analysis

PCR products, purified using exonuclease I/shrimp alkaline phosphatase (Exo-SAP-ITTM) (USB Corporation, Cleveland, OH), were sequenced in both directions using the same PCR primers in 10 µl

reactions, Big Dye™ chemistries, and an ABI 3100 sequencer analyzer (Applied Biosystems, Foster City, CA). Sequence chromatograms of each strand were aligned and examined with Lasergene software (DNASTAR, Inc., Madison, WI).

2.6. Determination of sensitivity of detection methods

To determine the sensitivity of the methods used to detect *Cryptosporidium* oocysts in the present study, bovine feces were obtained from a heifer found to be negative for cryptosporidiosis. Twenty five replicate 15 gm fecal specimens were each spiked with oocysts of *C. parvum* at the rate of 10, 50, and 100 oocysts per gram and subjected to the same methods of concentration and molecular detection as described above.

2.7. Statistical analysis

The prevalence of *Cryptosporidium* infection in 12–24-month-old heifers from the present study was compared with prevalence data for pre-weaned dairy calves and for post-weaned dairy calves from our previous studies on many of the same farms (Santín et al., 2004). The Chi-square test for independence was used to analyze the data and differences were considered very highly significant when $p \leq 0.0001$. The same test was applied to analyze prevalence data for 571 heifers at monthly intervals from 12 to 24 months of age. The prevalence of *Cryptosporidium* infection in 12–24-month-old heifers housed in confinement versus those on pasture was analyzed using Fisher's exact test. The same test was applied to analyze prevalence data for heifers on farms within each of these two groupings.

3. Results

3.1. Prevalence of *Cryptosporidium* by PCR and gene sequencing

PCR positive results for the 18S rRNA gene of *Cryptosporidium* were obtained for 13 of 14 farms (Table 1). For one farm (in North Carolina, NC-3) *Cryptosporidium* was not detected in any of 36 heifers. The percentage of positive specimens ranged from

3.4% for a farm in Virginia (VA-3) to 28.6% for another farm in Virginia (VA-2). The average prevalence for the 14 farms was 11.9%. *Cryptosporidium suis*, *C. parvum*, *Cryptosporidium* deer-like genotype, *C. bovis*, and *C. andersoni* were found on 1 (7.1%), 3 (21.4%), 5 (35.7%), 8 (57.1%), and 9 (64.3%) of the 14 farms, respectively. PCR positive results were obtained for 68 (11.9%) of the 571 fecal specimens (Table 2). Of these 68 specimens the following had 100% homology with genotypes listed in GenBank: 1 *C. suis* (previously reported as Pig 1 genotype) (GenBank accession number: [AF108861](#)), 4 *C. parvum* (GenBank accession number: [AF093490](#)), 10 *Cryptosporidium* deer-like genotype (GenBank accession number: [AY587166](#)), 24 *C. bovis* (previously reported as Bovine B genotype) (GenBank accession number: [AY120911](#) and [AY741305](#)), and 29 *C. andersoni* (GenBank accession number: [AB089285](#)).

The percentage of each species or genotype of *Cryptosporidium* relative to the total number of *Cryptosporidium*-positive heifers is shown in Fig. 1C. Of the 68 positive PCR specimens, *Cryptosporidium suis*, *C. parvum*, *Cryptosporidium* deer-like genotype, *C. bovis*, and *C. andersoni* constituted 1, 6, 15, 35, and 43%, respectively.

3.2. Prevalence of *Cryptosporidium*-positive heifers related to age

The prevalence of *Cryptosporidium*-positive heifers at each month of age from 12 to 24 months is shown in Fig. 1A. An overall increase in prevalence from 13 to 18 months of age is followed by a near absence of infection through 24 months of age. Despite the appearance of this trend the differences among months were not statistically significant. However, when prevalence data for all 571 12–24-month-old heifers were compared with data from 376 pre-weaned calves and 345 post-weaned calves from many of these same farms (Santín et al., 2004), differences in prevalence of *Cryptosporidium* among all three age groups were found to be very highly significant.

3.3. Prevalence of species related to age

The prevalence of each species and genotype of *Cryptosporidium* determined by PCR relative to the

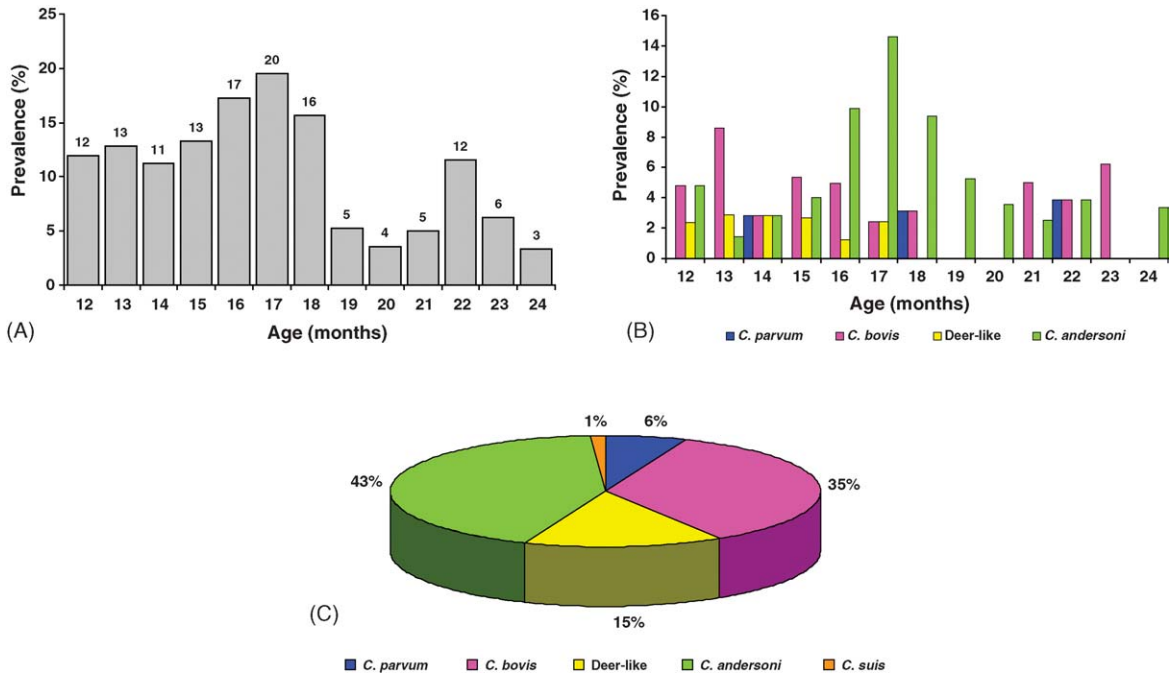


Fig. 1. (A). Prevalence of *Cryptosporidium*-positive heifers at each month of age from 12 to 24 months. (B). Prevalence of each species/genotype of *Cryptosporidium* determined by PCR relative to the age of the heifers. (C). Percentage of each species/genotype of *Cryptosporidium* relative to the total number of *Cryptosporidium*-positive heifers.

age of the heifers is shown in Fig. 1B. Of the 571 specimens examined, *C. suis*, *C. parvum*, *Cryptosporidium* deer-like genotype, *C. bovis*, and *C. andersoni* constituted 0.2%, 0.7%, 1.8%, 4.2%, and 5.1%, respectively. *Cryptosporidium andersoni* was not only the most prevalent species but was found in heifers throughout the 12–24 month age span. *Cryptosporidium bovis*, the second most prevalent species, was also found throughout this age span. In contrast, *C. parvum* was found only intermittently in four of the 571 heifers within this age span, and the *Cryptosporidium* deer-like genotype was found in <3% of heifers and was restricted to those that were 12–17 months of age.

3.4. Relationship between housing and prevalence of infection

Cattle on nine farms were confined in large fenced enclosures each with a cement floor and a roof covering one half or more of the floor area. Cattle on another farm (VA-2) were confined within a barn but had ad lib access to an adjacent open pen. The floors in

two enclosures were completely covered with soft feces that had accumulated to a depth of 10 cm or more, contained no clean bedding (VA-2 and NY-2), and there was no established cleaning schedule. Floors at the eight other farms were partially covered with feces and most had straw bedding under sheltered areas. These floors were scheduled to be scraped clean at approximately weekly intervals. Cattle on four farms (FL-1, FL-2, VA-3, and MD-2) were pastured on grass. All cattle were potentially exposed to insects, birds, rodents, and wildlife. Differences in prevalence of infection between confined and pastured heifers were not statistically significant, nor were differences in prevalence of infected heifers among farms within each of the two groups.

3.5. Sensitivity of detection method used in the present study

Of the 25 fecal specimens each spiked at 10, 50, and 100 oocysts per gram, 24%, 56%, and 84% were found positive by PCR, respectively.

4. Discussion

The results from molecular detection methods indicated the presence of *Cryptosporidium* on 13 of 14 farms and in all seven states where fecal specimens were collected along the East Coast of the United States from Vermont to Florida. Placement of cattle in confinement on cement flooring versus open fields made no statistically significant difference on the prevalence of infection. Approximately 12% of the specimens analyzed by PCR were found to be positive. However, the actual number of infected cattle is most likely underestimated based on findings with spiked specimens that demonstrated that not all specimens containing oocysts were found positive by the molecular methods used in this study. The sensitivity of the method increased as the number of oocysts in each spiked specimen increased, although the method did not detect 100% of the positive specimens at the highest rate of 100 oocysts per gram of feces.

The actual prevalence of infection in heifers is probably further underestimated because only one fecal specimen was collected per animal. If that specimen was identified as negative during a period when the calf was experiencing intermittent oocyst excretion, the calf would be considered negative. In comparison with younger cattle examined by virtually the same methods as in the present study, the prevalence of infection with *Cryptosporidium* in 12–24-month-old heifers was significantly lower ($p \leq 0.0001$) than the prevalence in post-weaned calves; and the prevalence of infection in post-weaned calves was significantly lower ($p \leq 0.0001$) than the prevalence in pre-weaned calves (Santín et al., 2004). The pattern of *Cryptosporidium* species versus age also showed change. In a study of calves 1 week to 11 months of age two peaks in prevalence were observed (Santín et al., 2004). The first peak (66.7%) was in 2-week-old calves and the second peak (30.4%) was in 6-month-old calves. The first peak was entirely *C. parvum*, whereas the second peak included *C. bovis*, *C. andersoni*, and the *Cryptosporidium* deer-like genotype. In calves older than 6 months the prevalence was much lower although *Cryptosporidium*-positive feces were obtained from calves on all 15 farms examined. This second peak was also observed in cattle in The Netherlands (Huetink et al., 2001). The reduction observed in prevalence of *Cryptosporidium* related

with the age agrees with most studies that reported the highest prevalence in animals of less than a month of age (Quílez et al., 1996; Sischo et al., 2000; Huetink et al., 2001; Sturdee et al., 2003). The continued significant decrease in prevalence with increasing age was observed in the present study with only 9 (5.6%) of 159 heifers infected from 19 to 24 months of age.

In the present study, no heifers were detected with a mixture of *Cryptosporidium* species or genotypes. However, the exponential nature of PCR often prevents detection of small *Cryptosporidium* subpopulations.

The findings that *C. bovis* and *Cryptosporidium* deer-like genotype were present and widespread in 12–24-month-old dairy cattle have not been reported previously. Only *C. parvum* and *C. andersoni* have been identified worldwide as species commonly infecting cattle (Enemark et al., 2002; Peng et al., 2003; Sakai et al., 2003) although *C. felis* (Bornay-Llinares et al., 1999) and *C. canis* (Fayer et al., 2001) have been reported to infect cattle. Most published prevalence studies of *Cryptosporidium* in cattle used microscopic methods incapable of identifying the species and genotypes infecting cattle (e.g. Xiao et al., 1993; Olson et al., 1997; Atwill et al., 1999; O'Handley et al., 1999; Fayer et al., 2000b; Wade et al., 2000; Castro-Hermida et al., 2002a; Kvac and Vitovec, 2003; Ralston et al., 2003). Molecular characterization of *Cryptosporidium* has helped to clarify the confusion in *Cryptosporidium* taxonomy and validate the existence of multiple species (Lindsay et al., 2000; Fayer et al., 2001; Morgan-Ryan et al., 2002; Xiao et al., 2004).

Of the three species and one genotype identified in the present study, only *C. parvum* is known to be zoonotic (Morgan et al., 1999). Therefore, when cattle are suspected as a source of cryptosporidiosis in humans, it is necessary to make a risk assessment. Based on the present findings 12–24-month-old dairy cattle are rarely infected with zoonotic *C. parvum* and any finding of *Cryptosporidium* must be confirmed at the molecular level. Reports of *Cryptosporidium* identified only at the genus level or identified as *C. parvum* or *C. parvum*-like based on oocyst morphology are insufficient for epidemiologic determinations.

The present study continued the documentation of the changing association of species and genotypes of *Cryptosporidium* with cattle as they age (Santín et al.,

2004), providing a guide for preventing, managing, and tracking sources of cryptosporidiosis. For example, in the present study *C. parvum* was detected from only 4 (0.7%) of the 571 heifers examined whereas it was the only species found in calves 1–2 weeks of age and it constituted 85% of positive specimens associated with all pre-weaned calves (Santín et al., 2004).

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